# Testicular parameters and spermatogenesis in different birthweight boars

# Testicular Parameters and Spermatogenesis in Different Birth Weight Boars

Auler, P.A.[*]; Moreira, G.H.F.A.[§]; Hogg, C.O.[£]; Ashworth, C.J.[£]; Bortolozzo, F.P.[†]; Chiarini-Garcia, H.[*]; Almeida, F.R.C.L.[*1]

[*]Biological Sciences Institute, Federal University of Minas Gerais, Av. Antônio Carlos 6627, 31.270-901, Belo Horizonte, MG, Brazil, [§]Veterinary School, Federal University of Minas Gerais, Av. Antônio Carlos 6627, 31.270-901, Belo Horizonte, MG, Brazil, [£]The Roslin Institute and R(D)SVS, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, UK, and [†]Faculty of Veterinary, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9090, 91.540-000, Porto Alegre, RS, Brazil

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

**ABSTRACT**

The present study investigated the impact of birth weight on testicular development and spermatogenesis in boars. Twenty four pairs of littermate boars were selected: one piglet with the highest (HW) and the other with the lowest birth weight (LW) within the litter. Two sub-sets of 12 pairs of male littermates from each experimental group were obtained after selection: one sub-set was orchiectomized at eight days and the other at eight months of age. HW boars had higher body and testicular weights at both ages ($P < 0.05$). Testosterone concentrations and the relative expression of 17-alpha hydroxylase in testis were similar between experimental groups. Birth weight affected somatic and germ cells numbers in the neonatal testis, which were higher in HW boars ($P < 0.05$). Moreover, a significant reduction in the number of pachytene spermatocytes and round spermatids was observed in LW boars ($P < 0.05$) at 8 months of age, which caused a decrease in the total number of elongated spermatids and daily sperm production ($P < 0.05$). Hence, HW boars have the potential to produce more sperm and consequently more semen doses per ejaculate, and would be very valuable to an industry that relies on artificial insemination.

**KEYWORDS:** testis, birth weight, boar, spermatogenesis

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

## INTRODUCTION

In the swine industry, both the number of offspring born and their developmental competence are critical. In this sense, breeding programs have focused on the increase of ovulation rate, however there was evidence that maternal limitations (uterine capacity) could affect both litter size and the average birth weight of the litter due to impaired placental growth and efficiency (Town *et al.* 2004; Wu *et al.* 2006).

Placental insufficiency affects nutrient and oxygen supply, impairing fetal development and growth (Pére and Etienne 2000; Wu *et al*. 2004; Town *et al*. 2004), which is severely aggravated in contemporary highly prolific commercial sows (Town *et al*. 2004). In fact, increased fetal number (uterine crowding) is not followed by an increase in uterine blood flow (Pére and Etienne 2000), which will lead to slowing of fetal growth and the birth of an individual with lower birth weight, which did not reach its full growth potential (Martin-Gronert and Ozanne 2006).

Low birth weight piglets are a reality in commercial farms and have been associated with functional disorders of several organs systems, resulting in deleterious consequences during postnatal life. There is strong evidence that low birth weight pigs present compromised postnatal growth and performance and poor meat quality (Gondret *et al*. 2006; Beaulieu *et al*. 2010; Alvarenga *et al*. 2013). However, reports of birth weight effects on the reproductive system are scarce, especially in boars (Almeida *et al*. 2009; Lin *et al*. 2015).

The use of artificial insemination (AI) for breeding pigs has been instrumental for facilitating global improvements in fertility, genetics, allocation of labour, and herd health. The establishment of AI centers for management of boars and production of

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

semen has allowed for selection of boars for fertility and sperm production using *in vitro* and *in vivo* measures (Knox 2016). With respect to the boar, increased genetic indices, fertility and high efficiency in the production of AI doses are main factors contributing to the high performance of pig production (Knox 2014). Given the importance of boars as semen donors in AI centers, it is essential to monitor fertility in these animals as they may represent a limiting factor for the improvement of reproductive efficiency of the breeding stock through the quality of the ejaculate (Waberski *et al*. 2008).

In this context, the effects of birth weight on testicular development and its implications on sperm production in boars deserves further investigation. If such effects exist, it would be essential to know if they could be identified at birth or would be apparent only later during their reproductive life. Therefore, the aim of the present study was to evaluate testicular parameters associated with spermatogenesis efficiency in different birth weight boars.

## MATERIAL AND METHODS

### *Animals and Experimental Design*

Forty-eight newborn male pigs Agroceres-PIC genotype (crossbred Landrace, Large White and Duroc) from 24 litters, born to $4^{th}$- $6^{th}$ parity sows, in litters of 10 to 15 total born, and mean litter birth weight from 1.25 to 1.65 kg, were selected immediately after birth, before they had suckled colostrum, and identified as falling into two birth weight categories: high (HW: birth weight range from 1.85 to 2.15 kg; n=24) and low (LW: birth weight range from 0.85 to 1.15 kg; n=24) littermates. The pair selected represented the highest and lowest birth weight boars from each litter. The criteria used at selection were based on the concept of intra-uterine crowding as performed in the study

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

of Alvarenga *et al.* (2013). Birth weight ranges for each experimental group were determined as mean +1SD to mean + 2SD for the HW and mean – 2SD to mean – 1SD for the LW groups, based on the average (mean) and standard deviation (SD) of birth weights previously obtained from 1,000 newborn piglets of the same genetic line. Litters containing runts, defined as piglets weighing less than 700 g, were avoided. Furthermore, in order to overcome possible litter birth weight effects on fetal development (Foxcroft *et al.* 2006), the piglets selected belonged to median birth weight litters, defined as the average litter birth weight registered at the farm in the previous year. Hence, the average litter birth weight range was 1.25 kg to 1.65 kg. At the end of selection, four experimental groups were obtained: two sub-sets of 12 pairs of male littermates from each experimental group which were bilaterally orchiectomized at eight days post-partum and two sub-sets of 12 pairs of male littermates from each experimental group, orchiectomized at eight months of age.

The surgical procedure used at both ages was the method of opened orchiectomy described by Turner and McIlwaith (2002). To perform orchiectomy in the 8 day-old boars, a local anesthetic (0.3 mL 2% lidocaine hydrochloride, Cristalia, Itapira, Brazil) was applied in the incision line. In the post-pubertal boars, surgical procedure was preceded by general anaesthesia using an intravenous injection of 2% xylazine hydrochloride (1.0 mg/kg, Bayer, Sao Paulo, Brazil) and10% ketamine (5.0 mg/kg, Agener União, Sao Paulo, Brazil). A local anesthetic (20 mL 2% lidocaine hydrochloride, Cristalia, Itapira, Brazil) was also applied in the incision line. The experiment was approved by the Ethical Committee in Animal Experimentation of the Federal University of Minas Gerais (protocol # 65/2011).

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

117 ***Biometrical data***

118 All pigs studied were weighed at birth and at orchiectomy. Immediately after
119 orchiectomy, testes were weighed without the epididymis and biometrical measurements,
120 including width, height, and length, were made to calculate testicular volume ($cm^3$),
121 assuming the shape of a prolate spheroid.

122

123 ***Tissue preparation***

124 Testicular samples were collected from the same area of the parenchyma (close to
125 the mediastinum) in the right and left testis in all animals with a razor blade and subjected
126 to different preparations according to the histomorphometrical, immunohistochemical,
127 gene expression, and sperm head count analysis. Samples of 1.0-2.0 mm thickness were
128 fixed through immersion in 5% glutaraldehyde in 0.05M phosphate buffer pH 7.3 for 24
129 hours, dehydrated in increasing concentrations of ethanol, embedded in glycol
130 methacrylate plastic resin (Historesin, Leica, Heidelberg, Germany), sectioned at 3 and 5
131 μm thicknesses and stained with toluidine blue sodium borate (Chiarini-Garcia *et al.*
132 2011), for all histomorphometrical evaluations. To perform immunohistochemistry,
133 samples were fixed in 4% paraformaldehyde in 0.05M phosphate buffer pH 7.3 for 24
134 hours and embedded in paraffin (Histosec, Merck, Darmstadt, Germany). Sections of 4
135 μm thickness were placed in silicanized slides.

136 For gene expression studies, fresh testes samples were preserved in RNA holder
137 (BioAgency, Sao Paulo, Brazil) for 24 hours overnight at 4°C and stored at -20°C.
138 Finally, for further sperm head count, other fresh testes samples were frozen at -20°C.

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[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

***Morphometric Methods***

For all the histomorphometrical evaluations, seven males were randomly selected within the subsets of each experimental group.

*Seminiferous cord/tubule diameter and seminiferous epithelium height*

Seminiferous cord (8 days) and tubule (8 months) diameter and seminiferous epithelium height (8 months) were measured using a graduated ruler fitted to an eyepiece of an Olympus BX 41 light microscope (Olympus, Tokyo, Japan) calibrated with a Leitz micrometer ruler. Ten round or nearly round seminiferous cords/tubules and their epithelium heights were measured in the 5 μm tissue sections from the right and left testes at a final magnification of 400X in 8-day old boars and 200X in the 8-month old ones.

*Volume density of the testicular components*

The volume densities (Vv%) of the testicular components (seminiferous cords/tubule and interstitium), tubular parameters (seminiferous epithelium, tunica propria and lumen) and germ (gonocytes at 8 days and type A spermatogonia at 8 months) and somatic (Sertoli and Leydig) cells were obtained using a 441-point grid placed in an eyepiece of the light microscope as described by Drumond *et al.* (2011a). Ten fields (total of 4410 points) were randomly selected per animal in the 5 μm tissue sections at 400X magnification.

*Cell counts per testis*

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

The absolute number of Sertoli, Leydig and germ cells (gonocytes/type A spermatogonia) per testis and per gram of testis were estimated based on their respective volume density obtained previously, according to the method described by Drumond *et al.* (2011a). The results are reported as total number of each cell type per testis and per gram of testis (ratio between the absolute number per testis and the testicular weight).

*Cell number and spermatogenesis efficiency*

All germ cell nuclei and Sertoli cell nucleoli present at stage I of the seminiferous epithelium cycle, according to the tubular morphology system (França and Cardoso 1998), were counted to evaluate spermatogenesis efficiency, as previously described by Melo *et al.* (2014).Ten round or nearly round cross-sections of seminiferous tubules were randomly selected per each animal at 1000X magnification. Cellular number per cross section was corrected for section thickness (5 μm) and nucleus diameter according to Abercrombie (1946) and modified by Amann and Almquist (1962). Nuclei diameter for each cellular type was obtained by the average of 10 nuclei per animal at 1000X magnification, using a graduated ruler fitted to an eyepiece and calibrated with a Leitz micrometer ruler. Due to their ovoid and non-round shape, the sizes of Sertoli cells nuclei at 8 days and type A spermatogonia nuclei at 8 months were obtained as the mean of their larger and smaller diameters. Using the correct cell counts present at stage I (type A spermatogonia, preleptotene primary spermatocytes, pachytene primary spermatocytes and round spermatids), the following ratios were obtained: (1) *mitotic index:* number of spermatocytes at preleptotene divided by the number of type A spermatogonia, to determine the coefficient of efficiency of spermatogonial mitosis; (2) *meiotic index*:

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

number of round spermatids divided by the number of pachytene spermatocytes, to obtain the rate of germ cell loss during meiosis; (3) *Sertoli cell efficiency:* number of round spermatids divided by the number of Sertoli cell nucleoli, to estimate the number of sperm supported by each Sertoli cell and (4) *spermatogenesis efficiency*: number of round spermatids divided by the number of type A spermatogonia at stage I, to determine the number of spermatids after mitotic and meiotic processes, and estimate the overall rate of spermatogenesis.

*Sperm counts and daily sperm production*

Homogenization-resistant testicular spermatids were counted as previously described by Drumond *et al.* (2011b). Approximately 0.1 – 0.2 grams of testis, without the albuginea tunica, was immersed in 1.0 mL of distillated water followed by sonication for 2 minutes (Cole Parmer Ultrasonic Processor, Illinois, USA), keeping the samples on ice. Sperm heads were counted in a Neubauer chamber (two fields per animal) with a 40X objective at phase contrast microscopy. Daily sperm production was estimated, as described by Okwun *et al*. (1996), dividing the number of elongated spermatids enumerated in the homogenate by 5.86, which is the number of days of the seminiferous epithelium cycle in which these spermatids are present in the seminiferous epithelium.

*Immunohistochemistry*

The spermatogonial proliferation activity was evaluated by the immunohistochemical detection of MCM7 (Minichromosome Maintenance Complex), a nuclear protein that is part of a complex essential for chromosomal DNA replication

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

(Pacek and Walter 2004). Slides were deparaffinized, rehydrated in decreasing ethanol concentrations and subjected to heat-induced antigen retrieval (water bath at 98°C, 30 min) with citrate buffer solution at pH 6.0. The slides were blocked with 30% of BSA (bovine serum albumin) at 0.3% in PBS for 60 min at 37ºC. All samples, except the negative controls, were subjected to overnight incubation (16 to 18h at 4ºC) with primary biotinylated antibody (mouse monoclonal anti-IgG anti-MCM7, clone 47DC141, 1:400 dilution, Abcam, Cambridge, United Kingdom). Negative control was maintained in PBS at 4ºC. In order to block the endogenous peroxidase activity, the slides were incubated with a solution of $H_2O_2$ (3%) in PBS for 30 minutes. All sections were incubated (30 min at 37ºC) with the secondary anti-mouse biotinylated antibody (1:500) (Vector Laboratories, Burlingames, California, USA), followed by incubation with avidin-streptavidin-peroxidase complex (Vector Laboratories, Burlingames, California, USA), both procedures for 30 minutes at 37ºC, and 3,30-diaminobenzidine tetrahydrochloride was used as a chromogen (DAB substrate system, Dakocytomation). Slides were counterstained with Mayer's hematoxylin and dehydrated in increasing ethanol concentrations. As positive control, samples of adult rat testis previously tested were used.

The proliferation activity index was calculated by the ratio between the number of positive cells and the total number of cells (positive + negative), in 30 cross sections of seminiferous tubules per animal. Because differentiation between preleptotene spermatocyte and type B spermatogonia is difficult after immunolabeling staining and both of them are placed in the same topographic position in the basal compartment,

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

proliferation activity of germ cells in 8 month old males was determined as the number of all labeled cells close to the basal membrane.

***Hormonal assay***

Five-mL blood samples were withdrawn from the jugular vein at orchiectomy (through venipuncture) in all 8 days and 8 months old boars for analysis of plasma testosterone concentrations. Blood samples were collected into heparinized tubes, centrifuged at 1,500 x g for 15 minutes, and plasma stored at -20$^{o}$ C until analysis.

Testosterone concentrations were quantified in duplicate, through electrochemiluminescence immunoassay "ECLIA" commercial kit (Roche Diagnostics USA, Indianapolis, USA) and used in COBAS E 411 immunoassay analyzers(Roche Diagnostics USA, Indianapolis, USA). The antibody used for the testosterone assay has less than 1% cross reactivity to other androgens. The sensitivity, estimated as 96.2% of total binding, was 1.0 ng/mL, and intra- and inter-assay CV were 15.0% and 6.3%, respectively.

***Total RNA extraction and cDNA synthesis***

In order to evaluate possible birth weight effects on sexual maturation, the expression of the steroidogenic enzyme 17alpha-hydroxylase (17a-OH), one of the enzymes in the steroidogenesis process which is responsible for the conversion of pregnenolone to testosterone, was measured by quantitative polymerase chain reaction (qPCR).

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

253 RNA was extracted from 20 - 50µg of tissue from each testis as previously
254 described by Hernandez *et al.* (2013). The RNA was quantified and the quality assessed
255 spectrophotometrically using a Nanodrop ND-1000 (Labtech International Ltd., East
256 Sussex, United Kingdom) and electrophoretically using a Tapestation 2200 (Agilent
257 Technologies LDA UK Limited, Cheshire, United Kingdom). The mean $A_{260}/A_{280}$ was
258 2.10 (range 2.06-2.16) and the mean RNA Integrity Number Equivalent ($RIN^e$) was 7.5
259 (range was 6.4 – 8.7). Extracted RNA was stored at -80ºC.
260 Complementary DNA (cDNA) was prepared from 1µg of each RNA with
261 SuperScript III reverse transcriptase (Life Technologies, Paisley, United Kingdom)
262 following the manufacturer's instructions. Each reaction contained 250 ng random
263 primers (Promega, Southampton, United Kingdom) and 40 units RNaseIn (Promega,
264 Southampton, United Kingdom). Negative controls without reverse transcriptase were
265 included in order to check for genomic contamination. Complimentary DNA was stored
266 at -20ºC.
267
268 ***Relative expression of 17a-OH in testis***
269 Quantitative PCR was performed on a Stratagene MX3000 instrument using
270 Platinum SYBR Green SuperMix UTG (Life Technologies, Paisley, United Kingdom).
271 The final concentrations of magnesium, ROX reference dye and each primer were 3mM,
272 50nM and 400nM, respectively. The reaction volume was 25µl. All qPCRs were carried
273 out at an annealing temperature of 60ºC and dissociation curves consisting of single
274 peaks were generated. Three reference genes were used: hydroxymethylbilane (HMBS),
275 succinate dehydrogenase (SDHA) and tyrosine 3-monooxygenase/tryptophan 5-

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

monooxygenase activation protein zeta polypeptide (YWHAZ). These had previously been selected (Ashworth *et al.* 2011) as stably expressed genes in pig testes tissue from a panel of nine candidates identified by Nygard *et al.* (2007) using geNORM V3.5 (Ghent University Hospital, Center for Medical Genetics). The primers sequences of the four genes used are shown in Table 1.

Serial dilutions of pooled cDNA ranging from 1:4 to 1:512 in nuclease-free water were used as standards. Sample cDNA was diluted 1:20 and 5µl of sample, standard or control were added per well. Each plate contained duplicate wells of a no template control (NTC), standards, one of two sets of sample cDNA and reverse transcriptase blanks (RTBs). Data was analyzed using qbase$^{+}$ softwareV3.0 (Biogazelle, Zwijnaarde, Belgium). A target and run specific strategy was employed and the results, normalized to the three reference genes, are scaled to a representative sample. The mean slope, intercept, PCR efficiency and $R^2$ values are shown in Table 2.

***Statistical analysis***

All variables measured were tested for normality prior to analyses, using the univariate procedure of the Statistical Analysis System (SAS Institute, 2001). Data were analyzed as a randomized complete block design, each block consisting of two littermates. The statistical model included birth weight class and block as fixed factors and boar as random factor. Treatment effects on biometrical parameters, histomorphometrical analysis, immunohistochemical analysis, testosterone concentrations, and the relative expression of 17a-OH in the testis were analyzed using the general linear model (GLM) procedure of SAS. Least square means were compared

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

using the Student's t-test with $P < 0.05$ being considered significant. In the tables and figures, data are reported as least square means and the pooled SEM. Potentially relevant associations among characteristics measured were examined across treatment groups using correlation analysis (INSIGHT procedure of SAS).

## RESULTS

### *Body weight changes and testicular measurements*

Body weight changes in both sub-sets (8 days and 8 months) from birth to the time of orchiectomy are shown in Table 3. Body weight differences observed at birth were maintained until the time of orchiectomy, as LW animals had lower body weights compared to their HW littermates in both sub-sets ($P < 0.05$).

Testicular weight and volume were also affected by birth weight, which was shown by the lower values in LW compared to HW boars (Table 3) at both ages evaluated. Interestingly, testis weight relative to body weight (gonadossomatic index – GSI: testis weight divided by body weight X 100) was similar between both experimental treatments at the ages studied, demonstrating a proportional relationship between body weight and testicular weight.

A litter of origin effect was evident for testicular weight, GSI and testicular volume at 8 days ($P < 0.05$), which revealed the importance of the use of littermates when designing experiments of this kind to account for the differences due to family. Moreover, at 8 days of age, testicular weight was highly correlated with birth weight ($r = 0.59$, $P < 0.01$) and body weight at castration ($r = 0.73$, $P < 0.01$) and Sertoli cell number

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

per testis (r = 0.65; P = 0.012); body weight was also positively correlated with Sertoli cell number per testis (r = 0.56; P = 0.04).

***Histomorphometrical analysis and spermatogenic parameters***

Seminiferous cord/tubule diameter and the number of Sertoli cells per cross section of seminiferous cord/tubule were not affected by birth weight class in both ages studied. However, LW animals presented a significant reduction in seminiferous epithelium height compared to their HW counterparts at 8 months of age (P < 0.05; Table 3).

The volumetric density of the testicular parenchyma components in both ages studied is shown in Table 4.The percentage of interstitium, seminiferous cord/tubule were similar between groups at both ages, as well as the percentage of seminiferous tubule components in 8 month-old boars. Additionally, the volumetric density of somatic (Sertoli and Leydig) and germ (gonocytes at 8 days and type A spermatogonia at 8 months of age) cells were similar between groups at both ages. However, the numbers of Sertoli, Leydig and germ cells (gonocytes at 8 days)per testis were lower at 8 days old LW boars (P < 0.05), which was not observed in LW males at 8 months of age. Despite the differences in total cell numbers per testis at 8 days-old, when these parameters were calculated per gram of testis the results were similar between both experimental groups (Table 4).

Additionally, low birth weight was not associated with depletion in spermatogenesis efficiency, represented by mitotic, meiotic and Sertoli cell efficiency indexes (Fig.1). Further evidence of normal spermatogenesis efficiency was established

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

by counting type A spermatogonia (HW: 1.0 ± 0.3; LW: 0.8 ± 0.2) and preleptotene spermatocyte (HW: 23.4 ± 2.2; LW: 20.7 ± 4.1) per seminiferous tubule cross section, which were similar between both experimental groups. Notwithstanding the lack of treatment effect on spermatogonia A and preleptotene spermatocyte, a significant reduction in the number of pachytene spermatocyte and round spermatid was observed in LW boars ($P < 0.05$ – Table 5), that caused a decrease in the total number of round spermatids, the most mature spermatogenic cells at stage I of the seminiferous epithelium cycle.

***Plasma testosterone concentration and relative expression of 17a-OH in the testes***

Birth weight did not affect plasma testosterone concentrations or the relative expression of 17a-OH in the testes, as represented by similar values for both parameters evaluated between experimental groups at both ages (Table 3).

***Sperm counts and daily sperm production***

Sperm counts, determined after tissue sonication, and daily sperm production were affected by birth weight as LW boars presented lower total spermatids number. However, when this number was adjusted for testicular weight (spermatid number per gram of testis), both experimental groups showed similar values.

Interestingly, lower daily sperm production was observed in the LW males compared to HW littermates ($P < 0.05$; Fig. 2). Moreover, a litter of origin effect was also observed for spermatids number and daily sperm production ($P < 0.05$).

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

***Cellular proliferation activity***

Cellular proliferation activity, measured by the percentage of MCM7-stained cells relative to the total number of cells, was not affected by birth weight. Both treatment groups showed similar proliferation activity for Sertoli cells (89.8 ± 2.6 vs 94.8 ± 2.6 %), Leydig cells (24.4 ± 3.0 vs 26.1 ± 3.0 %), and gonocytes (63.0 ± 6.0 vs 62.4 ± 6.0 %;), respectively for HW and LW 8-day old boars (Fig.3A).

As Sertoli cells do not proliferate in post-pubertal boars, proliferation activity was measured in Leydig cells, and type A spermatogonia in the 8 month-old subset. Again, the proliferation activity at this age was not affected by birth weight, as observed by the similar values obtained for Leydig (6.3 ± 1.2 vs 7.8 ± 1.2 %) and type A spermatogonia (98.5 ± 0.6 vs 98.5 ± 0.6 %) cellular proliferation, respectively in HW and LW boars (Fig.3B).

## DISCUSSION

Many studies have investigated the effects of birth weight on postnatal growth performance, and yet there is a lack of information on subsequent reproductive performance of low birth weight males. As birth weight may be an important parameter to include in sire line breeding programs, a better understanding of the impact of birth weight on fertility seems critical. Hence, the present study investigated the effects of birth weight on testicular development and spermatogenesis in littermate boars. In particular, it was shown that birth weight affects spermatogenesis leading to a decrease in sperm production. This is believed to be the first report showing that low birth weight alters the spermatogenic process in male pigs.

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

Similar to previous studies (Beaulieu *et al*. 2010; Alvarenga *et al*. 2013; Lin *et al*. 2015) where growth rate of different birth weight pigs was investigated, LW boars showed lower body weight at castration in both ages studied. Moreover, testicular weight and volume were also affected by birth weight, which was also reported by Almeida *et al*. (2009) and Smit *et al*. (2013) in neonatal males and by Lin *et al*. (2015) in adult boars. Despite the differences in body and testes absolute weights, the gonadossomatic index, which is an indicator of the testicular relative weight, was similar between both experimental groups, suggesting that testes size is proportional to body size (Table 3). The correlation between birth weight and body weight at castration ($r = 0.73$, $P < 0.01$) provides strong evidence of their dependence. Furthermore, the results of proliferation activity obtained for somatic and germ cells suggest that testis growth was progressing at similar intensity in both experimental groups at either 8 days or 8 months of age.

On the other hand, the reduction of testicular weight and volume in LW boars may not be related to the structural organization of the testicular parenchyma. Since there is a proportion between testicular tissue components and organ size, as shown by the similarities in volumetric density and number of cells per gram of testis between the experimental groups, birth weight may not be associated with impaired testicular organogenesis. Hence, the components and cells of the testicular parenchyma in LW animals are proportional to their smaller size.

Studies considering different breeds have shown that testis weight and volume are highly correlated to the number of Sertoli cells and this to sperm production in post-pubertal boars (Okwun *et al*. 1996; Ren *et al*. 2009). In fact, LW pigs presented a reduced number of Sertoli and Leydig cells and gonocytes compared to HW group at 8 days of

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

413 age, which was also shown in the study of Smit *et al*. (2013). Some studies demonstrated
414 that Sertoli cells can support a relatively fixed number of germ cells depending on the
415 species, for instance rabbits, rats, and monkeys (Russell and Peterson 1984; Orth *et al*.
416 1988). Therefore, the number of Sertoli cells established during testis development until
417 puberty may be a limiting factor for sperm production in adulthood (Orth *et al*. 1988).
418 Nevertheless, at 8 months, the differences in testis weight and volume could not be
419 explained by the number of somatic and germ cells present in the testis, which were
420 similar in both experimental groups.

421 Compromised fetal growth did not affect spermatogenic efficiency, as also
422 described by Melo *et al*. (2014) in rats submitted to protein deficiency *in utero*. However,
423 in contrast to the present study, Melo *et al*. (2014) observed a reduction in Sertoli cell
424 support capacity. Our results also show that germ cell death and proliferation activity,
425 which are important to the regulation of spermatogenic cell population (França *et al*.
426 2005), were not affected by compromised fetal growth.

427 Another important parameter for evaluating spermatogenic efficiency is
428 seminiferous tubular diameter, which is also related to the number of Sertoli cells per
429 cross section of seminiferous tubule and epithelium height (França and Russell 1998). In
430 the present study, LW boars did not show changes in tubular diameter and the number of
431 Sertoli cell per cross section, which is in agreement with the findings reported by Lin *et*
432 *al*. (2015) in adult boars. However, a reduction in epithelium height was observed in LW
433 boars. Despite the similarities in tubular diameter, the differences in epithelium height
434 can be associated with a decrease in germ cell number present in seminiferous tubule at
435 stage I of the epithelium cycle. In fact, a reduction in the total number of pachytene

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

436 spermatocyte and round spermatids per cross section of seminiferous tubule was
437 demonstrated in the present study, which did not alter tubular diameter but affected
438 epithelium height in LW males. We believe that the decrease in the number of
439 spermatids, present in seminiferous tubule cross sections, may be related to a further
440 decrease in sperm production as germ cell division follows a geometric progression. Even
441 though a small numeric difference was observed in the early germ cells stages it became
442 more pronounced overtime, reaching statistical significance in the later stages. This
443 difference still remained for elongated spermatid number and daily sperm production in
444 8-month old boars. Assuming that sperm concentration in the ejaculate would be
445 proportional to the daily sperm production in the testis, HW boars would produce
446 approximately 34% more semen doses, based on the data presented herein (daily sperm
447 production: HW – 122 x $10^6$ vs LW – 80 x $10^6$ per testis per day). Actually, Lin *et al.*
448 (2015) provided evidence of deleterious effects of prenatal programming on sperm
449 production in intra-uterine growth restricted boars, whereas the present results
450 demonstrated negative effects of birth weight on germ and somatic cells population in small,
451 but perfectly formed piglets.

452 Similar testis expression of 17a-OH and plasma testosterone concentrations in LW
453 and HW boars at 8-days and 8-months of age suggest that sexual maturation may not be
454 compromised by altered fetal growth, as this enzyme, which catalyses the production of
455 precursors for glucocorticoid, estrogen and androgen synthesis, is involved in sexual
456 development during fetal life and at puberty (Majdic *et al*. 1996). The absence of birth
457 weight effects on circulating testosterone levels were also reported in 10 months old
458 boars (Lin *et al*. 2015).

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

Taken together, our results suggest that low birth weight is associated with a decrease in testicular somatic and germ cell numbers in the neonatal period. During the post-pubertal period, low birth weight affected sperm production. The reductions in biometrical measures and somatic and germ cell numbers shown in the present study did not seem to originate from compromised organogenesis and function, but were proportional to the smaller size of the animal. However, HW boars produce more sperm and consequently more semen doses per ejaculate. As semen from elite boars is in huge demand around the world, elite boars that produce more sperm per ejaculate would be very valuable to an industry that relies on AI. Hence, the selection of potential AI boars of high birth weight would be predictive of better lifetime productivity in the boar stud.

Assuming that these results will be confirmed at the multiplication level in sire-line selection programs, the implications of birth weight for lifetime sperm production seem real. This suggests that prenatal programming of testis development will predetermine the reported relationship between adult testis size and lifetime semen production. Therefore, additional studies are necessary to better understand the effects of birth weight on other reproductive parameters related to semen quality and fertility.

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[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

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[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

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640 **Tables**

641 **Table 1**. Porcine-specific primer sequences for qPCR

| Primer names | Sequence(5'– 3') | Ampliconsize (bp) | Tm (ºC) | Accession numbers |
|---|---|---|---|---|
| **17α-OH** | | 202 | 60 | M63507 |
| Forward | CTGTGGGCAAGGAAATTTTG | | | |
| Reverse | ACTTTCTGCGTTCGTCTTGG | | | |
| **HMBS2** | | 83 | 60 | DQ845174 |
| Forward | AGGATGGGCAACTCTACCTG | | | |
| Reverse | GATGGTGGCCTGCATAGTCT | | | |
| **SDHA** | | 141 | 60 | DQ845177 |
| Forward | CTACAAGGGGCAGGTTCTGA | | | |
| Reverse | AAGACAACGAGGTCCAGGAG | | | |
| **YWHAZ** | | 203 | 60 | DQ845179 |
| Forward | TGATGATAAGAAAGGGATTGTGG | | | |
| Reverse | GTTCAGCAATGGCTTCATCA | | | |

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[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

**Table 2.** qPCR calibration curve data

| Gene | Slope | Intercept | Efficiency | $R^2$ |
|---|---|---|---|---|
| **17αOHase** | -3.33 | 17.33 | 100.6 | 0.995 |
| **HMBS** | -3.51 | 28.02 | 92.7 | 0.998 |
| **SDHA** | -3.218 | 29.71 | 104.6 | 0.992 |
| **YWHAZ** | -3.245 | 21.172 | 103.5 | 0.996 |

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

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668 **Table 3.** Body and testicular biometry, tubular, hormonal and molecular parameters of 8
669 day and 8 month high (HW) and low (LW) birth weight littermate boars

670

| PARAMETERS | 8 days | | 8 months | |
|---|---|---|---|---|
| | HW | LW | HW | LW |
| Body weight at birth (kg) | 1.8 ± 0.1[a*] | 1.0 ± 0.1[b] | 1.9 ± 0.1[a] | 1.0 ± 0.1[b] |
| Body weight at castration (kg) | 3.6 ± 0.1[a] | 2.3 ± 0.1[b] | 176 ± 3.6[a] | 158 ±3.6[b] |
| Testisweight (g) | 2.4 ± 0.1[a] | 1.4 ± 0.1[b] | 413 ± 15[a] | 355 ± 16[b] |
| Gonadossomatic index | 0.07 ± 0.003[a] | 0.06 ± 0.003[a] | 0.23 ± 0.01[a] | 0.23 ± 0.01[a] |
| Testis volume ($cm^3$) | 3.9 ± 0.2[a] | 2.3 ± 0.2[b] | 676± 30[a] | 575 ± 32[b] |
| Cord/tubulediameter (μm) | 48 ± 1.2[a] | 51 ± 1.2[a] | 248 ± 5.3[a] | 246 ± 5.3[a] |
| Seminiferousepitheliumheight (μm) | - | - | 92 ±5.3[a] | 81 ± 6.2[b] |
| Testosterone (ng/mL) | 2.4 ± 0.6[a] | 2.4 ± 0.6[a] | 3.9 ± 0.9[a] | 3.4 ± 0.9[a] |
| 17α- hydroxylase mRNA expression | 9.0 ± 2.2[a] | 7.8 ± 1.8[a] | 1.8 ± 0.6[a] | 1.6 ± 0.5[a] |

671 [a,b]Within a row and age sub-set, lsmeans without a common superscript differ ($P < 0.05$).

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[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

679 **Table 4.** Volume density (Vv%) of testicular parenchyma components and number of
680 somatic and germ cells at 8 days and 8 months of age in high (HW) and low (LW) birth
681 weight boars

| | 8days | | 8months | |
|---|---|---|---|---|
| | **HW** | **LW** | **HW** | **LW** |
| **Testicular parameters (%)** | | | | |
| Interstitium | 76.4 ± 5.7[a*] | 78.6 ± 4.8[a] | 28.8 ± 4.7[a] | 28.9 ± 6.7[a] |
| Seminiferouscord/tubule | 23.6 ± 5.3[a] | 21.4 ± 4.8[a] | 71.2 ± 5.0[a] | 71.1 ± 6.0[a] |
| **Seminiferostubule (%)** | | | | |
| Epithelium | - | - | 54 ± 10[a] | 53.4 ± 6.0[a] |
| Tunica propria | - | - | 6.2 ± 0.6[a] | 6.2 ± 0.9[a] |
| Lumen | - | - | 11 ± 2.0[a] | 11.5 ± 2.0[a] |
| **Sertolicells** | | | | |
| Volume density (%) | 9.4 ± 2.2[a] | 8.5 ± 2.0[a] | 1.4 ± 0.3[a] | 1.5 ± 0.3[a] |
| Number ($10^9$)/testis | 1.2 ± 0.1[a] | 0.6 ± 0.1[b] | 86 ± 12[a] | 98 ± 12[a] |
| Number ($10^6$)/g of testis | 500 ± 64[a] | 429 ± 64[a] | 208 ± 26[a] | 276 ± 26[a] |
| **Leydig cells** | | | | |
| Volume density (%) | 6.3 ± 1.2[a] | 7.0 ± 1.1[a] | 2.1 ± 0.7[a] | 2.2 ± 0.6[a] |
| Number ($10^9$)/testis | 0.6 ± 0.1[a] | 0.3 ± 0.1[b] | 4.3 ± 0.4[a] | 4.3 ± 0.4[a] |
| Number ($10^6$)/ g of testis | 250 ± 42[a] | 214 ± 42[a] | 10.4 ± 1.8[a] | 12.1 ± 1.8[a] |
| **Gonocyte/A spermatogonia*** | | | | |
| Volume density (%) | 0.7 ± 0.2[a] | 0.6 ± 0.2[a] | 0.5 ± 0.2[a] | 0.6 ± 0.2[a] |
| Number ($10^8$)/ testis | 0.1 ± 0.1[a] | 0.06 ± 0.1[b] | 41 ± 11[a] | 30 ± 10[a] |
| Number ($10^6$)/ g of testis | 4.2 ± 0.3[a] | 4.3 ± 0.3[a] | 10 ± 1.8[a] | 8.5 ± 1.8[a] |

[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

682 [a,b] Within a row and age sub-set, lsmeans without a common superscript differ (P < 0.05).

683 *Gonocytes and type A spermatogonia were scored at 8 days and 8 months of age, respectively.

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688 **Table 5.**Germ cell numbers per cross section in high (HW) and low (LW) birth weight

689 boars, present at stage I of the seminiferous epithelium cycle, at 8 months of age

690

| PARAMETERS | HW | LW |
|---|---|---|
| A spermatogonia | 1.0 ± 0.1[a] | 0.9 ± 0.1[a] |
| A spermatogonia/g of testis | 0.002 ± 0.001 [a] | 0.003 ± 0.001 [a] |
| Preleptotene spermatocyte | 23.3 ± 2.1[a] | 20.6 ± 4.1[a] |
| Preleptotene spermatocyte/g of testis | 0.06 ± 0.01[a] | 0.06± 0.02[a] |
| Pachytene spermatocyte | 25.6± 3.9 [a] | 20.0± 2.9[b] |
| Pachytene spermatocyte/g of testis | 0.06 ± 0.002 [a] | 0.06± 0.001[a] |
| Round spermatid | 72.4 ± 14.0[a] | 59.7 ± 7.3[b] |
| Round spermatid/g of testis | 0.18 ± 0.03[a] | 0.18 ± 0.04[a] |

691 [a,b] Within a row, lsmeans without a common superscript differ (P < 0.05).
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[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br

698 **Figure Legends**

699 **Fig.1**- Spermatogenesis efficiency in high (HW) and low (LW) birth weight boars,
700 calculated based on germ cell counts present at stage I of the seminiferous epithelium
701 cycle at 8 months of age.

702

703 **Fig. 2**- Sperm count and daily sperm production (DSP) of high (HW) and low (LW) birth
704 weight boars at 8 months of age ($^{a,b}$ $P < 0.05$).

705

706 **Fig. 3** - Immunostaining of MCM7-positive cells in 8 days (A) and 8 months (B) boars.
707 Ai and Bi correspond to negative control. G, gonocyte; Se, Sertoli cell; S, spermatogonia;
708 L, Leydig cell. Bars: 10 µm.

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[1]Corresponding author address: Av. Antonio Carlos, 6627; e-mail: falmeida@icb.ufmg.br